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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A selected number of immunobiologically active polypeptides have been found to be closely associated with, but separable from the lipopolysaccha- ride endotoxin (LPS) in the outer membrane of Gram-negative bacteria. Initially these endotoxin associated proteins (EP) from <u>Bordetella pertussis</u> , <u>Salmonella typhi</u> and <u>Vibrio cholerae</u> were found to enhance the immune response to cholera enterotoxin after immunization with cholera toxoid. At the cellular level, <u>B. pertussis</u> EP (PEP) is a mitogen and polyclonal		

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20. Abstract (Continued)

activator of antibody producing B-lymphocytes of C3H/HeJ mouse lymphocytes which are unresponsive to LPS. PEP can adjuvant in vitro the production of IgM antibody to cholera toxin and sheep erythrocytes by mouse splenic lymphocytes. Studies are continuing to determine whether macrophages and T-lymphocytes are required for the enhancement of antibody production (IgM and IgG) by EP in both primary and secondary responses.

In control experiments we have shown that the activity of PEP cannot be neutralized by the cationic polypeptide polymyxin B which specifically neutralizes the lipid A component of LPS that is responsible for the stimulating properties of LPS. In addition, our tests indicate PEP does not contain any detectable lymphocytosis promoting factor (LPF) activity. LPF from B. pertussis can act as an adjuvant particularly for homocytotropic antibodies. The outer membrane PEP may also act as a protective immunogen against a lethal challenge of B. pertussis.

Preliminary experiments have shown that extracts of B. pertussis which contain both LPS and associated proteins are protective in the standard mouse model used for testing the efficacy pertussis vaccines. The protective effects of LPS and PEP are currently under study.

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During the first year of this project, we have compared the polypeptide complexes from several strains of B. pertussis which we refer to as pertussis endotoxin protein (PEP). The studies described include analysis by polyacrylamide gel electrophoresis and experiments on the immunobiological activities of these molecules.

A. Comparative strain analysis of PEPs. The comparison of the endotoxin proteins from four different strains of B. pertussis was initiated to determine whether there were differences in the polypeptide profiles obtained from each strain and whether such differences might be expressed in the immunopotentiating and immunogenic activities of these materials. The strains selected included the NIH 114 (phase I) with which our preliminary studies were conducted, strain 18323 (phase I), which is used as the virulent challenge organism in the standard protection assays used by the FDA for evaluating the efficacy of pertussis vaccines and strain 11615 (phase IV), which is an avirulent mutant. In addition, we have joined with Dr. Alvin Winters of the University of Alabama in a collaborative effort to evaluate the chemistry and biological activity of preparations from the vaccine strain 10536 (Connaught Laboratories) which he has used to induce protection against viral infections in mice. Dr. Winters has prepared a lipopolysaccharide-protein extract by the trichloroacetic acid method (Boivin) we have used routinely. Further separation of the polypeptide complex has been done in our laboratory as well as a preliminary analysis of both products.

Separate lots of PEP from NIH 114 grown in shaker cultures showed similar polypeptide profiles on SDS-PAGE. Coomassie blue staining bands appeared at 31, 33, 44, 48 and 64 KD and a low molecular blue at about 12 KD. Two static grown cultures produced PEP with lesser amounts of the same polypeptides except for the 64 KD bands. These values differed somewhat from our very first preparation most likely due to different gel conditions of the PAGE system. Dr. Winters' material showed a similar profile of 31, 33, 37, 41, 48 and 64 KD; however, the PEP from the challenge strains 18323 demonstrated a triplet of 33, 35 and 37 KD and 2 minor bands between 67 and 94 KD. All of the preparations were mitogenic for C3H/HeJ LPS low responder mice including # 18323. The NIH 114 is an adjuvant and polyclonal activator. The remaining samples are to be tested for adjuvanticity and the preparation of the PEP from the mutant 11615 is in progress. Until all of the biological data are developed, we cannot draw any conclusions as yet.

B. Polymyxin B Effects on PEP. The influence of this cationic polypeptide antibiotic has been studied to determine whether the immunomodulating activities are due to any residual LPS present in the PEP preparations. Polymyxin B has been shown to specifically neutralize the lipid A component of LPS which is responsible for most of the toxic and stimulating properties of LPS. Two methods have been used to neutralize LPS activity with polymyxin B; (a), the binding of polymyxin B with LPS or PEP at a 3:1 ratio with subsequent dialysis to remove unbound polymyxin B and (b), direct addition of polymyxin B to mouse lymphocyte cultures incubated with LPS or EP. Similar results were obtained by both methods. Measurements of mitogenic activity and polyclonal activation by plaque forming cells (PFC) against TNP coated sheep red blood cells were made.

Representative data using the direct addition method are shown in Tables 1 and 2.

TABLE 1. Mitogen Response

LPS/EP Source	Reactants (ug/ml)	Percent Inhibition of ³ H-Tdr incorporation	
		LPS	EP
<u>S. typhi</u>	50 LPS/EP + 10 PB	89.0	13.1
	10 LPS/EP + 10 PB	93.5	0.0
<u>B. pertussis</u>	50 LPS/EP + 10 PB	75.0	0.0
	10 LPS/EP + 10 PB	93.0	3.8

TABLE 2. PFC Response

LPS/EP Source	Reactants (ug/ml)	Percent Inhibition of Nos. of PFC	
<u>S. typhi</u>	10 LPS/EP + 10 PB	81.0	0.0
	10 LPS/EP + 10 PB	86.6	8.5
<u>B. pertussis</u>	10 LPS/EP + 5 PB	60.0	14.9
	10 LPS/EP + 10 PB	86.8	19.3

Within the experimental systems, these results demonstrate that the activity of the EPs as mitogens and polyclonal activators cannot be inhibited to any appreciable extent by polymyxin B, whereas significant inhibition by polymyxin B approaches complete neutralization in some cases and was obtained from the LPS from which the EPs were dissociated.

C. LPF activity of PEP. LPF or pertussis toxin, as it is now called, has been shown to be a potent activator of various cell types. This material has been purified from extracellular filtrates of B. pertussis cultures and found to be a single homogeneous protein. LPF can be associated with the bacilli and, therefore, we considered it to be a possible contaminant of our PEP preparations. This is of particular concern since LPF can act as an adjuvant particularly in the production of homocytotropic (IgE) antibodies.

One of the most sensitive assays for the detection of LPF activity is to measure the increase in leukocytes (primarily lymphocytes) in the blood of mice 3 days after being given various doses of LPF intravenously. Representative results are shown in Table 3.

TABLE 3.

Material	Dose per mouse	Mean Total	
		WBC (mm ³)	Lymphocytes (mm ³)
LPF (Jap NIH)	0.1 µg	49,468	37,407
	1.0 µg	35,537	29,519
PEP (114)	10.0 µg	6,433	5,229
	50.0 µg	7,707	6,231
Saline	-	8,866	6,649

The results of these assays clearly show that the PEP at doses 100 to 500 times that of LPF causes no discernible increase in total leukocytes or lymphocytes in the blood of mice. It should be noted that preparations of *S. typhi* EP are likewise inactive indicating that there is no inherent activity in the Gram-negative outer membrane proteins associated with the LPS in producing a leukocytosis. Therefore, we conclude that the immunoadjuvant activity we have observed in previous studies is not due to any residual LPF that could have contaminated the PEP.

D. Immunoregulatory Activities of PEP at the Cellular Level. Previous studies with *Salmonella typhi* EP demonstrated that the polypeptides are potent mitogens for B-lymphocytes and can stimulate polyclonal antibody by B-cells in culture. Although the polypeptides from PEP differed from *S. typhi*, their adjuvant activity in vivo has been shown to be equivalent or better. Likewise, PEP is a potent mitogen for mouse spleen cells including the C3H/HeJ LPS low responder lymphocytes. We have shown PEP can activate C3H/HeJ splenic lymphocytes to make IgM antibody to TNP-SRBC in contrast to pertussis LPS which was ineffective (1,2). However, this polyclonal effect was reduced as much as 75% when the spleen cells were depleted of macrophages (2). These results suggest that macrophages may play a role as an augmenting cell but may not be a required accessory cell for polyclonal activation of B-cells by PEP. Furthermore, PEP can adjuvant the production of specific antibody to cholera toxin to a greater extent than pertussis LPS in CF-1 mouse spleen cells in vitro, which are LPS responsive. In a similar manner, PEP can adjuvant specific direct plaque forming cells to SRBC after 5 days of culture.

(1) Kinetics of the adjuvant effect in vitro. A more detailed examination of the effect of PEP in vitro has been conducted using the T-dependent antigen SRBC to determine the effect of timing on adjuvanticity. Representative data are shown in Table 4.

TABLE 4. The PFC response to SRBC in CF-1 splenic lymphocytes

Stimulant	Mean PFC/10 ⁶ viable cells					
	After 3 Days			After 5 Days		
	-1	0	+1	-1	0	+1
Control	50	40	10	21	201	141
PEP	104	329	438	186	573	445

These results indicate that the optimal adjuvant effect in vitro is obtained when PEP is added simultaneously (0) with antigen in culture. However, significant enhancement remains when PEP is added a day after the cells are cultured with the antigen (+1). Although an adjuvant effect is obtained when PEP is added prior to the antigen (-1), a significant decrease is seen as compared to the optimal response. Although suppressor cell activity could be invoked as an explanation, it remains to be determined whether true suppression is obtained under these conditions.

(2) Effect of Accessory Cells on Adjuvanticity Induced by EP. Our initial experiments were designed to determine whether macrophages were necessary for EP stimulated adjuvanticity observed in cultured splenic lymphocytes. Macrophage depletion has been achieved by repeated (3 to 4 times) adsorption on carbonyl iron. The remaining cell population contained approximately 1% macrophages although only 40 to 50% of the total lymphocytes were recovered. When these lymphocytes are cultured at high density (10×10^6 cells/ml) with TNP-SRBC as the antigen and optimal concentrations of LPS or EP, the reduction of specific PFC as compared to undepleted cultures is minimal varying from 0 to 14%. The EP used in these experiments was derived from S. typhi. Future experiments will test the effects of macrophage depletion on PEP. However, it would appear that under these conditions macrophages are not essential for the adjuvant effect. Nevertheless, complete removal of macrophages has not been achieved nor has complete recovery of the initial lymphocytes after treatment occurred so that this conclusion is tentative at best. Other methods have been used including plastic adherence and column separation; however, the depletion of macrophages was less complete or the reduction of total lymphocytes was greater so that we have not used these methods or combination of methods for the adjuvant experiments. Future studies will employ low density cultures to reduce the absolute numbers of macrophages present. Additionally, experiments to determine the effect of T-cell depletion have begun.

To date all of our adjuvant experiments in vitro have been conducted in the presence of fetal calf serum. However, initial trials have shown that serum-free RPMI-1640 medium supplemented with insulin, transferrin and progesterone is effective in supporting polyclonal activation by EP and adjuvanticity as well, so that comparative experiments can be run to determine whether any factors in serum are essential for PEP stimulation.

E. Immunogenicity of PEP. Given the fact that the polypeptides of EP are present in the outer membrane of Gram-negative bacteria, it seemed reasonable that they may act as protective immunogens. Indeed, in our previously published work, Salmonella typhimurium EP has been shown to protect CD-1 mice against a challenge of 500 LD₅₀s of S. typhimurium and was more effective than the LPS from this organism. For these reasons and the need for more effective and less toxic components for a pertussis vaccine, we have initiated experiments to determine whether PEP can provoke specific protection against an infective challenge with B. pertussis.

We have adopted the standard protection assay prescribed for testing the efficacy of the whole cell pertussis vaccine now used in the United States, as a method for determining whether outer membrane components of B. pertussis are protective. Using CF-1 mice, we have set about to standardize conditions of the assay so that comparisons could be made between the accepted whole cell vaccine (US8) from the FDA and the experimental immunogens. Over a 5 month period, a series of LD₅₀ measurements were made of the B. pertussis challenge which produced an average LD₅₀ of $10^{-5.42}$ ($10^{-5.32}$ to $10^{-5.55}$) where 10^0 represents a suspension of B. pertussis (18323) with a reading of 62% T at 640 nm.

Protection experiments have been carried out using the US8 vaccine in conjunction with several potential immunogens. In three trials, the 50% protective dose endpoint (PD₅₀) was achieved with a mean PD₅₀ = 10^{-2.4} (10^{-1.89} to 10^{-3.0}).

An example of our latest trial demonstrates that the Boivin type antigen from B. pertussis is protective at microgram levels.

TABLE 5. Immunogenicity of B. pertussis Boivin antigen

Vaccine	Dilution/Concentration	Survivors/Total	Percent Survival
US8	10 ⁻¹	11/11	100
	10 ^{-1.7}	10/10	100
	10 ^{-2.4}	7/9	77.7
	10 ^{-3.1}	6/11	54.5
Boivin (10536) ¹ .	250 µg/ml	11/11	100
	50 µg/ml	11/11	100
	10 µg/ml	11/11	100
	2 µg/ml	9/11	81.8
Boivin (18323) ² .	250 µg/ml	11/11	100
	50 µg/ml	11/11	100
	10 µg/ml	10/10	100
	2 µg/ml	6/11	54.5
Saline ³ .	-	0/11	0

1. TCA extract prepared from Connaught strain of B. pertussis by A. Winters
2. TCA extract prepared from challenge strain of B. pertussis in our lab.
3. The challenge inoculum was 87 LD₅₀s in this trial.

These results show that Boivin antigens from B. pertussis, which consist of an LPS-EP complex, are highly protective. Further trials will be conducted using the separated components individually and together to determine the active moieties, although according to the literature, purified LPS is not a protective antigen against pertussis.

While these experiments were in progress, we also took advantage of the surviving mice which were bled for serum antibody and/or given booster immunizations and rebled. In addition, two groups of mice were given a series of injections with (1), live B. pertussis i.p. which is non-lethal by this route and (2), the US8 vaccine so that we now have a panel of 7 sera which have been used in our ELISA assay as shown below.



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TABLE 6. ELISA of mouse antisera to pertussis antigens

Antisera	Titer ^a .	
	PEP	Boivin
Normal serum	1: 6,000	1: 4,000
2679	1:15,000	1:15,000
2680	1:26,000	1:12,000
2693	1:29,000	1:29,000
2715	1:20,000	1:15,000
2716	1:16,000	1:13,000
2717 ^b .	1:20,000	1:13,000
2718 ^c .	1:50,000	1:35,000

a. endpoint taken at OD of 0.6 at 410 nm.

b. immunized with live B. pertussis.

c. immunized with US8 vaccine.

Clearly, live or dead whole cells of B. pertussis generate antibody to the envelope components. Use of this panel of antibodies for identifying the protective antigens by immunoblot measurements of SDS-PAGE separated polypeptides is now in progress.

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Fritz H. Bach, M.D.
Director, Immunology Research Center
University of Minnesota
Box 724, Mayo Memorial Bldg.
420 Delaware St., SE
Minneapolis, MN 55455

Francis A. Ennis, M.D.
Department of Medicine
University of Massachusetts
Medical School
55 Lake Avenue
Worcester, MA 01605

Fred D. Finkelman, M.D.
Department of Medicine
Uniformed Services University
of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814

Dr. Matthew J. Kluger
Department of Physiology
University of Michigan Med. School
7620 Medical Science II Bldg.
Ann Arbor, MI 48109

Dr. Vijaya Manohar
Borrison Laboratories, Inc.
5050 Beech Place
Temple Hills, MD 20748

Dr. Ernest D. Marquez
Bioassay Systems Corporation
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Woburn, MA 01801

James J. Mond, M.D.
Department of Medicine
Uniformed Services University
of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814

Dr. Donna S. Sieckmann
Infectious Diseases Program Center
Naval Medical Research Inst.
National Naval Medical Center
Bethesda, MD 20814

Dr. John D. Clements
Department of Microbiology
and Immunology
Tulane University Medical Ctr.
1430 Tulane Avenue
New Orleans, LA 70112

Dr. Edward A. Havell
Trudeau Institute
P.O. Box 59
Saranac Lake, NY 12983

Dr. Arthur G. Johnson
Department of Medical
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University of Minnesota
School of Medicine
2205 East 5th Street
Duluth, MN 55812

Dr. Philip Lake
Immunologic Oncology Division
Lombardy Cancer Center
Georgetown Univ. School of Med.
Washington, DC 20007

W. John Martin, M.D., Ph.D.
Laboratory, Dept. of Medicine
Naval Hospital
National Naval Medical Center
Bethesda, MD 20814

Dr. Robert I. Mishell
Dept. of Microbiology &
Immunology
Univ. of California, Berkeley
Berkeley, CA 94720

Dr. Page S. Morahan
Department of Microbiology
Medical College of Pennsylvania
3300 Henry Avenue
Philadelphia, PA 19129

Dr. Alan L. Schmaljohn
Department of Microbiology
University of Maryland
School of Medicine
660 W Redwood Street
Baltimore, MD 21201

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Dr. Jeannine A. Majde, Code 441CB
Scientific Officer, Immunology Program
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David A. Stevens, M.D.
Department of Medicine
Santa Clara Valley Medical Center
Stanford University
751 S. Bascom Avenue
San Jose, CA 95128

Dr. Barnet M. Sultzter
Department of Microbiology &
Immunology
Downstate Medical Center
450 Clarkson Avenue
Brooklyn, NY 11203

Dr. Alvin L. Winters
Department of Microbiology
University of Alabama
University, AL 35486

Dr. Phyllis R. Strauss
Department of Biology
Northeastern University
360 Huntington Avenue
Roston, MA 02115

G. Jeanette Thorbecke, M.D.
Department of Pathology
New York University
School of Medicine
550 First Avenue
New York, NY 10016

Lyn Yaffe, M.D.
Research Support Center
Naval Medical Research Inst.
National Naval Medical Center
Bethesda, MD 20814